ELSEVIER

Biochimica et Biophysica Acta 1539 (2001) 181–191

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Palmitoylation of the canine histamine H2 receptor occurs at Cys^{305} and is important for cell surface targeting

Yasushi Fukushima [a,*], Toshihito Saitoh [b], Motonobu Anai [a], Takehide Ogihara [a], Kouichi Inukai [a], Makoto Funaki [a], Hideyuki Sakoda [a], Yukiko Onishi [a], Hiraku Ono [a], Midori Fujishiro [a], Takashi Ishikawa [a], Kuniaki Takata [c], Ryozo Nagai [a], Masao Omata [a], Tomoichiro Asano [a]

[a] *Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Tokyo 113-8655, Japan*

[b] *Department of Internal Medicine, Tokyo Women's Medical University Daini Hospital, 2-1-10 Nishiogu, Arakawa-ku, Tokyo 116-8567, Japan*

[c] *Department of Anatomy and Cell Biology, Gunma University School of Medicine, Maebashi, Gunma 371-8512, Japan*

Received 2 January 2001; received in revised form 9 April 2001; accepted 9 April 2001

Abstract

To determine the presence and functional role of the histamine H2 receptor (H2R) palmitoylation, a receptor with a Cys^{305} to Ala (A^{305} receptor) mutation was generated. Wild-type (WT) and A^{305} receptors were tagged at their N-termini with a hemagglutinin (HA) epitope. WT, but not A^{305}, receptors incorporated [^{3}H]palmitate by metabolic labeling, indicating that the H2R is palmitoylated at Cys^{305}. Immunocytochemistry of WT and A^{305} receptors expressed in COS7 cells revealed WT receptors to be distributed at the plasma membrane, while the majority of A^{305} receptors were localized intracellularly with only a small portion being at the plasma membrane. However, the affinity of the A^{305} receptor for tiotidine was comparable to that of the WT receptor. In addition, when the amounts of cell surface receptors as determined by anti-HA antibody binding were equivalent, A^{305} receptors mediated production of more cAMP than WT receptors. Preincubation of COS7 cells expressing each receptor with 10^{-5} M histamine for 30 min reduced subsequent cAMP production in response to histamine via the receptors to similar extents, indicating that palmitoylation is not necessary for desensitization. In addition, cell surface A^{305} receptors were capable of being internalized from the cell surface at a rate and extent similar to those of WT receptors. Finally, CHO cell lines stably expressing either WT or A^{305} receptors were incubated with 10^{-5} M histamine for 1, 6, 12 and 24 h. Total amounts of WT and A^{305} receptors, as determined by tiotidine binding, were reduced by incubation, indicating downregulation. Downregulation of the A^{305} receptor was more extensive than that of the WT receptor. Thus, palmitoylation of the H2R might be important for targeting to the cell surface and stability.

Keywords: Histamine H2 receptor; Palmitoylation; Subcellular localization; Site-directed mutagenesis

Abbreviations: HA, hemagglutinin; PBS, phosphate-buffered saline; GPCR, G protein-coupled receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; BSA, bovine serum albumin

* Corresponding author. Fax: +81-3-5803-1874 E-mail: fksm@tm.kcom.ne.jp

0167-4889/01/$ – see front matter
PII: S0167-4889(01)00104-5

1. Introduction

Several G protein-coupled receptors (GPCRs) are reportedly modified by palmitate at highly conserved Cys residues located in the C-terminus [1–10]. Palmitoylation of GPCRs has been reported to be involved in agonist-induced internalization [5], and agonist-induced downregulation [11]. In addition, GPCR palmitoylation has been implicated in the regulation of adenylyl cyclase stimulation via the β_2-adrenoceptor by rendering its C-terminus relatively inaccessible to receptor kinases [12,13]. Thus, the roles of GPCR palmitoylation vary among receptors. Cys residues at corresponding positions are conserved in other GPCRs including the histamine H2 receptor (H2R) [14–16]. To date, no data have been presented concerning palmitoylation of H2R. The purpose of this study was, therefore, to examine whether the canine H2R, which contains a Cys residue at position 305 in the C-terminal region, is palmitoylated and, if so, to assess its functional roles. Herein, using a mutant H2R in which Cys^{305} had been replaced with Ala, we were able to demonstrate that the H2R is palmitoylated at Cys^{305}. Using this mutated receptor, we attempted to elucidate the role of palmitoylation in cAMP production via adenylyl cyclase as well as the intracellular distribution of the receptor.

2. Experimental procedures

2.1. Materials

[^{3}H]Palmitate was obtained from Moravek. 12CA5 monoclonal antibody against the hemagglutinin (HA) nine-amino epitope was purchased from Boehringer Mannheim (Germany), ^{125}I-goat anti-mouse IgG from ICN, and pBacPAK9 from Clontech (Palo Alto, CA, USA).

2.2. Construction of palmitoylation-defective H2R cDNA

Full-length canine H2R cDNA was subcloned into the *Eco*RI site of pUC19. A mutant H2R cDNA ($C^{305} \rightarrow A$, A^{305}) was prepared by site-directed mutagenesis. Mutagenesis was performed by polymerase chain reaction using 25-mer (GCTCTTCCGCGCGAGGCCGGCCAGC) and 25-mer (TGGCCGGCCTGGCGCGGAAGAGCTG). Then, a nine-amino acid HA epitope (YPYDVPDYA) was inserted into the N-termini of the WT and mutant receptors. The cDNAs encoding the WT H2R and its mutants with or without the HA epitope were subcloned into pCAGGS. COS7 cells were transfected with varying amounts of plasmid DNAs by the DEAE–dextran method. Transfection into Chinese hamster ovary (CHO) cells was performed employing the calcium phosphate precipitation method. Several clones resistant to 600 μg/ml G418 were isolated [17].

2.3. Production of recombinant H2R baculovirus

For expression in Sf9 cells, *N*-glycosylation-defective H2Rs, HA-$Q^{4,162,168}$ and its mutant HA-$Q^{4,162,168}A^{305}$, were used. *N*-Glycosylation-defective H2Rs migrated as a sharp band on immunoblotting of membrane fractions from COS7 cells or CHO cells and were easier to identify [18]. The cDNAs were subcloned into pBacPAK9, a baculovirus vector. The recombinant baculoviruses were produced according to the manufacturer's instructions. Sf9 cells were grown as monolayers or in suspension culture in TC100 medium containing 10% fetal calf serum and 50 μg/ml kanamycin at 27°C. Sf9 cells were infected with the appropriate virus at a multiplicity of infection of 2–5 and harvested at 48 h post infection.

2.4. Immunoprecipitation and immunoblotting of H2Rs

At 48 h post infection, Sf9 cells expressing HA-$Q^{4,162,168}$ or HA-$Q^{4,162,168}A^{305}$ receptors were lysed in 1% Triton X/phosphate-buffered saline (PBS) with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.1 mg/ml bacitracin, 0.1 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor and 100 units/ml aprotinin. The samples were centrifuged at $10\,000 \times g$ at 4°C for 10 min, and the supernatants were then incubated with 2 μg of anti-HA monoclonal antibody, 12CA5, conjugated to protein G Sepharose at 4°C for 2 h. The immunocomplexes recovered by centrifugation were washed five times with PBS containing 1% Triton X-100, then boiled in Laemmli sample buffer. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel

electrophoresis (SDS–PAGE) and then transferred to nitrocellulose filters. The filters were incubated with antibody against the 14 C-terminal amino acids of the H2R (aH2R^{CT} antibody) [17]. The proteins were visualized by enhanced chemiluminescence (ECL) using horseradish peroxidase-labeled anti-rabbit IgG.

2.5. Labeling of infected Sf9 cells with [^{3}H]palmitate

Sf9 cells infected with the baculoviruses containing cDNAs encoding HA-Q^{4,162,168} and HA-Q^{4,162,168}A^{305} receptors were cultured for 18 h prior to the labeling experiment in serum free medium containing 0.1% fatty acid free bovine serum albumin (BSA). The serum free medium was replaced with serum free medium containing 0.5 mCi/ml [^{3}H]palmitate and 0.1% fatty acid free BSA and incubated at 27°C for 4 h. Then, the cells were washed twice with ice-cold PBS and lysed in PBS containing 1% Triton X. Next, the samples were subjected to immunoprecipitation with anti-HA monoclonal antibody, 12CA5, and SDS–PAGE. Then, the gels were placed in fixative (40% methanol/10% acetic acid/3% glycerol) for 4 h and then immersed in either 1 M Tris–HCl, pH 7.0 or 1 M hydroxylamine, pH 7.0 for 12 h at room temperature. After washing with 2-propanol/water (1:1, v/v), the gels were exposed to Kodak X-Omat films at −80°C for 30 days.

2.6. Quantification of cell surface H2Rs

At 24 h post transfection, COS7 cells expressing HA-WT and HA-A^{305} receptors or parental COS7 cells were plated onto 24-well plates at a density of 10^5 cells/well. At 48 h post transfection, cells were incubated in HEPES–tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 5.6 mM glucose, 25 mM HEPES, pH 7.4) containing 0.1% BSA at 37°C for 30 min. After incubation in the presence or absence of 10^{-5} M histamine at 37°C for the indicated times, the cells were fixed with PBS containing 3% paraformaldehyde, washed again with PBS and incubated with 5% skim milk/PBS for 1 h at room temperature. They were incubated with 200 μl of 12CA5 (5 μg/ml) in PBS for 2 h at room temperature, washed again with PBS, and incubated for one additional hour with 200 μl of ^{125}I-goat anti-mouse IgG (1/200 dilution) at room temperature. The wells were then washed twice with 5% skim milk/PBS, twice with PBS, and three times with 0.05% Tween 20/PBS. Bound ^{125}I-goat anti-mouse IgG was solubilized in 1% SDS, and radioactivities were determined in a γ-counter. Specific binding was determined by subtraction of the non-specific binding observed in parental COS7 cells.

2.7. Tiotidine binding assay

Tiotidine binding in membranes was assessed as described previously, with some modifications [17]. Membrane fractions (200 μg) from COS7 cells were incubated with 1 nM [^{3}H]tiotidine and varying concentrations of unlabeled tiotidine in 25 mM HEPES, 0.1% BSA, pH 7.4, in a final volume of 200 μl at 37°C for 2 h. The binding reaction was terminated by filtration over GF/C glass fiber filters, followed by 10 ml washes with ice-cold incubation buffer. Radioactivity on the filters was determined by liquid scintillation counting. Specific binding was calculated by subtraction of the non-specific binding determined in the presence of 10^{-4} M ranitidine. No specific binding was observed in either parental COS7 cells or COS7 cells transfected with the expression vector alone. Similar experiments were performed in CHO cells stably expressing WT or A^{305} receptors.

2.8. Measurement of cAMP productions

COS7 cells, plated onto 24-well plates, were assayed at a density of 1×10^5 cells/well, as described previously [18,19]. The cells were incubated for 30 min at 37°C in 450 μl of HEPES–tyrode's buffer containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine, then 50 μl of histamine solution were added to initiate the reaction. After 10 min of incubation at 37°C, the reaction was terminated by adding 500 μl of 12% trichloroacetic acid. The samples were centrifuged for 5 min at $3000\times g$ at 4°C. Following extraction of the supernatants three times with diethyl ether, cAMP contents in the samples were measured using a radioimmunoassay. Histamine-dependent and forskolin-dependent cAMP productions were determined by subtracting basal cAMP productions.

2.9. Immunocytochemical localization of H2R in COS7 cells

For immunofluorescence microscopy, COS7 cells were fixed in 3% paraformaldehyde/PBS, scraped off the dish with a rubber blade, and embedded in 10% gelatin/PBS. Semithin frozen sections (1 μm thick) were cut and incubated with anti-H2R^{CT} antibody. These sections were then incubated with rhodamine-labeled affinity purified goat anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) [20].

3. Results

3.1. Palmitoylation of the H2R

To determine the occurrence of H2R palmitoylation, we made use of the baculovirus/Sf9 expression system, which allows efficient expression of receptors. In addition, we utilized *N*-glycosylation-defective H2Rs, which retain the functions of the normal WT receptor and migrate as a sharp band on SDS–PAGE [18]. The receptor tagged with the HA epitope at its N-terminus (termed HA-Q^{4,162,168} receptor) and recombinant baculoviruses were produced. At 48 h post transfection the infected Sf9 cells were lysed and immunoprecipitated with 12CA5, a monoclonal antibody against the HA epitope. Successful expression of the HA-tagged receptors is shown in Fig. 1A (lane 2). There are at least four immunoreactive bands with molecular masses of 31.5 ± 2.5 kDa, 59.0 ± 6.0 kDa, 80.5 ± 4.5 kDa and 120 kDa, respectively. The differences in molecular mass are not due to different extents of *N*-glycosylation, because the receptors we used were devoid of all potential *N*-glycosylation sites (Asn^4, Asn^{162} and Asn^{168}). Rather, these bands apparently correspond to oligomers of H2Rs as we have previously reported [21]. Sf9 cells, infected with HA-Q^{4,162,168} receptor virus, were isotopically labeled with [^{3}H]palmitate, lysed in PBS containing 1% Triton X, and the receptors were immunoprecipitated with anti-HA monoclonal antibody. As shown Fig. 1B lane 3, ^{3}H-labeled bands with molecular masses corresponding to the immunoprecipitated HA-

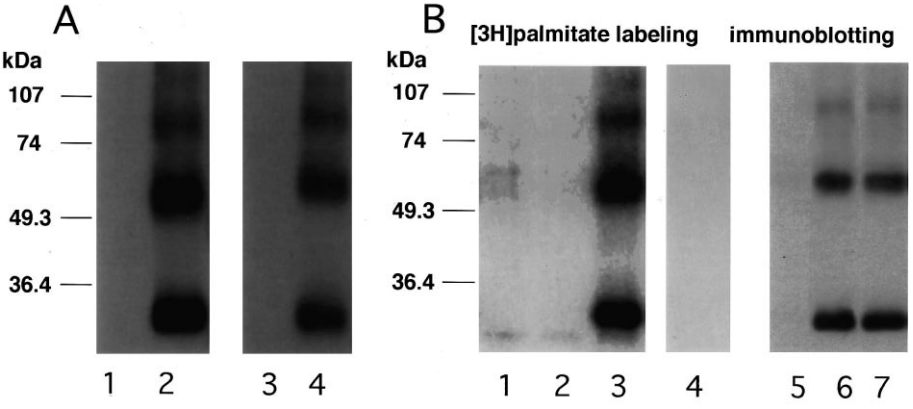

Fig. 1. (A) Expression of H2Rs in Sf9 cells. Parental Sf9 cells and Sf9 cells infected with the HA-Q^{4,162,168} and HA-Q^{4,162,168}A^{305} receptor recombinant baculoviruses, respectively, were harvested, solubilized in 1% Triton X buffer, and immunoprecipitated with 2 μg of anti-HA monoclonal antibody conjugated to protein G Sepharose as described in Section 2. Samples were subjected to SDS–PAGE and transferred to nitrocellulose filters. H2R proteins were detected using anti-H2R^{CT} antibody and the ECL system. Lanes: 1 and 3, parental Sf9 cells; 2, HA-Q^{4,162,168} receptor; 4, HA-Q^{4,162,168}A^{305} receptor. (B) Cys^{305} to Ala mutation eliminates detectable [^{3}H]palmitate incorporation into the canine H2R. Parental Sf9 cells and Sf9 cells infected with HA-Q^{4,162,168} receptor and HA-Q^{4,162,168}A^{305} receptor recombinant baculoviruses, respectively, were metabolically labeled in the presence of 0.5 mCi/ml [^{3}H]palmitate for 4 h at 27°C. Immunoprecipitation was performed as described in Section 2. The samples were subjected to SDS–PAGE and the gels were dried and exposed to a Kodak X-Omat film at −80°C for 30 days. Lanes: 1, parental Sf9 cells; 2, HA-Q^{4,162,168}A^{305} receptor; 3, HA-Q^{4,162,168} receptor; 4, HA-Q^{4,162,168} receptor with hydroxylamine treatment. Immunoblotting was also performed with the immunoprecipitated samples using anti-H2R^{CT} antibody and the ECL system. Lanes: 5, parental Sf9 cells; 6, HA-Q^{4,162,168}A^{305} receptor; 7, HA-Q^{4,162,168} receptor. The results are typical of three experiments.

$Q^{4,162,168}$ receptor were observed. In addition, neutral hydroxylamine treatment removed the [^{3}H]palmitate incorporated into HA-$Q^{4,162,168}$ receptors, suggesting that palmitoylation occurs on a Cys residue. This shows that H2R is a palmitoylation substrate (Fig. 1B, lane 4). To determine whether palmitoylation takes place at the conserved Cys^{305}, we constructed a cDNA for a mutant H2R, the Cys^{305} of which had been replaced by Ala (HA-A^{305}). As shown in Fig. 1A (lane 4), HA-$Q^{4,162,168}A^{305}$ receptors were expressed in Sf9 cells. However, no ^{3}H labeling was observed following [^{3}H]palmitate labeling of the Sf9 cells infected with the HA-$Q^{4,162,168}A^{305}$ virus (Fig. 1B, lane 2). The difference in ^{3}H labeling between the HA-$Q^{4,162,168}$ and the HA-$Q^{4,162,168}A^{305}$ receptors cannot be explained by diminished uptake or incorporation of [^{3}H]palmitate by HA-$Q^{4,162,168}A^{305}$ cells, since SDS–PAGE of total cell homogenates from both cell types revealed identical incorporation of ^{3}H label into total cellular proteins (data not shown). These findings suggest that the H2R is palmitoylated at Cys^{305}. Neither can decreased HA-$Q^{4,162,168}A^{305}$ receptor expression account for the difference in ^{3}H labeling (Fig. 1B, lane 6).

3.2. Subcellular localization of WT and A^{305} receptors

To investigate the functional significance of palmitoylation of the H2R, WT and A^{305} receptors were transiently expressed in COS7 cells. Fusion genes encoding the WT and A^{305} receptors tagged at their N-termini, with the HA epitope, were prepared and subcloned into the mammalian expression vector pCAGGS. These receptors as well as those without the HA epitope were expressed in COS7 cells. Immunocytochemistry of COS7 cells showed both WT and HA-WT receptors to be distributed mainly at the plasma membrane (Fig. 2A). In contrast, the majority of A^{305} and HA-A^{305} receptors were distributed intracellularly with only small portions at the plasma membrane (Fig. 2A). The difference in subcellular distribution between WT and A^{305} receptors was not due to a difference in expression level, because comparable amounts of each receptor type were expressed in COS7 cells based on tiotidine binding (Fig. 2B). Furthermore, HA tagging at the N-terminus did not affect the normal distribution of the receptors. Thus, the difference in subcellular dis-

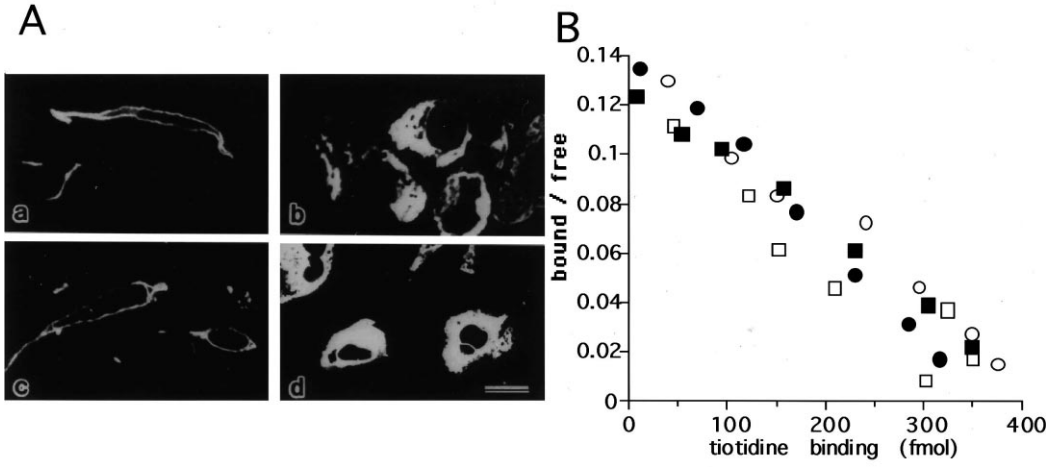

Fig. 2. (A) Immunocytochemical localization of WT and A^{305} receptors in COS7 cells. COS7 cells on 10 cm dishes were transfected with 3 μg of each plasmid DNA (a, WT; b, A^{305}; c, HA-WT; d, HA-A^{305} receptor). At 48 h post transfection, COS7 cells were fixed in 3% paraformaldehyde/PBS. Thin sections were prepared and immunostained with anti-HA antibody (c,d) or anti-$H2R^{CT}$ antibody (a,b). Scale bar, 10 μm. (B) Scatchard plot analysis of the tiotidine binding data for WT and A^{305} receptors. COS7 cells on 10 cm dishes were transfected with 3 μg of each plasmid DNA (WT, A^{305}, HA-WT and HA-A^{305} receptors). At 48 h post transfection, COS7 cells were harvested and membrane fractions prepared. Membranes (200 μg) thus prepared were incubated with 1 nM [^{3}H]tiotidine and increasing concentrations of unlabeled tiotidine at 37°C. Non-specific binding was determined in the presence of 10^{-4} M ranitidine. Data shown are representative of three experiments performed in duplicate. ○, WT receptor; □, A^{305} receptor; ●, HA-WT receptor; ■, HA-A^{305} receptor.

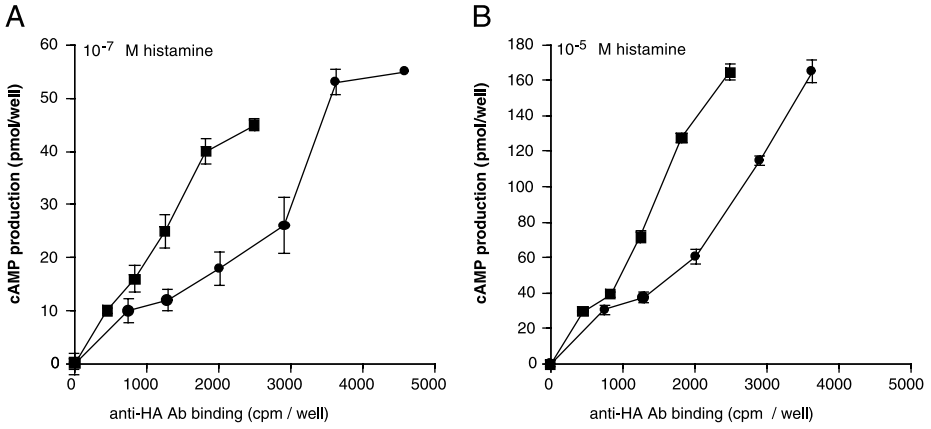

Fig. 3. Relationship between the numbers of cell surface receptors expressed and cAMP productions via WT and A^{305} receptors. At 48 h post transfection, COS7 cells expressing HA-WT or HA-A^{305} receptors on 24-well plates at a density of 10^5 cells/well were preincubated in HEPES–tyrode's buffer containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine for 30 min, then incubated for an additional 10 min in the absence or presence of 10^{-7} M (A) or 10^{-5} M (B) histamine. Incubation was terminated by adding trichloroacetic acid. cAMP contents were measured by radioimmunoassay. ●, HA-WT; ■, HA-A^{305}. Data (mean ± S.E.) are from one of three representative experiments performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

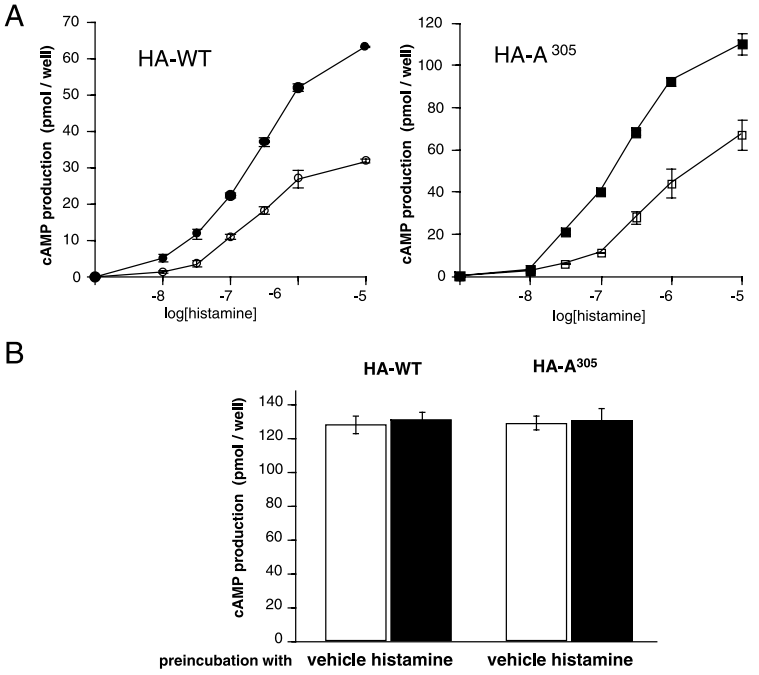

Fig. 4. Effect of histamine treatment on subsequent cAMP accumulation. COS7 cells were transfected with plasmid DNAs containing HA-WT (0.3 μg/10 cm plate) or HA-A^{305} (1.5 μg/10 cm plate) receptors. Transfection with these DNA amounts resulted in similar levels of cell surface expression of HA-WT and HA-A^{305} receptors. At 48 h post transfection, COS7 cells expressing HA-WT or HA-A^{305} receptors were preincubated in the presence or absence of 10^{-5} M histamine for 30 min at 37°C. Subsequently, the cells were washed and either varying concentrations of histamine (A) or 10^{-4} M forskolin (B) were added to the cells, which had been preincubated with histamine. Incubations were continued for an additional 10 min at 37°C and reactions were then terminated by adding trichloroacetic acid. ●,■, preincubation with vehicle; ○,□, preincubation with histamine. Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

tribution of the palmitoylation-defective receptors was attributable to lack of palmitoylation.

3.3. Adenylyl cyclase coupling of the WT and A^{305} receptors

We next attempted to define the role of palmitoylation in H2R coupling to adenylyl cyclase. Plasma membrane localizations of WT and A^{305} receptors differed markedly, making it difficult to compare the abilities of the receptors to produce cAMP in response to histamine even when the total amounts of expressed receptors were similar. cAMP productions mediated by these receptors can be compared only when the amounts of cell surface receptors per cell are equivalent. Furthermore, measurement of the amounts of cell surface H2Rs is extremely difficult. The acid wash method [22] was not applicable to H2R due to high levels of non-specific uptake of histamine and [^{3}H]histamine. In addition, there are no appropriate hydrophilic radioligands specific for the H2R [23]. Thus, to quantitatively determine the amounts of cell surface WT and A^{305} receptors, the amounts of cell surface anti-HA antibody binding were measured. Various amounts of plasmid DNAs for HA-WT and HA-A^{305} receptors were transfected into COS7 cells. Importantly, the amount of plasmid DNA affected the number of H2Rs expressed per cell and the percentage of transfected cells was constant even when the amounts of plasmid DNA used were not equivalent, as reported previously [24]. cAMP productions in response to histamine at each expression level in COS7 cells expressing HA-WT and HA-A^{305} receptors are shown in Fig. 3. Interestingly, the HA-A^{305} receptor was more potent in terms of stimulating adenylyl cyclase than the HA-WT receptor when cell surface receptor expression per cell was equivalent. Basal levels of cAMP production were similar between HA-WT and HA-A^{305} receptors (not shown). Thus, palmitoylation of the H2R is not essential for functional coupling of this receptor to adenylyl cyclase stimulation. Although the percentage of cell surface receptors was not determined, functional coupling of the palmitoylation-defective receptor to adenylyl cyclase was also observed in CHO cell lines stably expressing A^{305} receptors (not shown).

3.4. Role of palmitoylation in homologous desensitization, agonist-induced internalization, and agonist-induced downregulation

We examined the effect of preincubation of HA-WT and HA-A^{305} cells with 10^{-5} M histamine on subsequent cAMP production. COS7 cells were transfected with plasmid DNAs containing HA-WT (0.3 μg/10 cm plate) or HA-A^{305} (1.5 μg/10 cm plate) receptors. Transfection with these DNA amounts resulted in similar levels of cell surface expression of HA-WT and HA-A^{305} receptors. Preincubation of transfected COS7 cells with 10^{-5} M histamine for 30 min led to reduced cAMP productions in both HA-WT and HA-A^{305} cells as compared with untreated cells (Fig. 4A), indicating desensitization, while forskolin-dependent cAMP production was not altered (Fig. 4B). Since the transfection efficiency of the COS7 cell system is approx. 20% at best, the results shown in Fig. 4B do not necessarily mean that forskolin-dependent cAMP production in COS7 cells expressing the receptors was not altered. Therefore, we performed similar experiments in CHO cells stably expressing HA-WT or HA-A^{305} receptors and found that histamine-dependent cAMP production via these receptors was reduced by preincubation with 10^{-5} M histamine, while forskolin-dependent cAMP production was unaffected (not shown). These results suggest that palmitoylation of the H2R is not essential for desensitization of the receptor-mediated cAMP response. Similar results were obtained employing a 15 min incubation with 10^{-5} M histamine.

We have already shown HA-WT receptors to be rapidly internalized from the cell surface after histamine exposure [20]. We examined the role of palmitoylation in agonist-induced internalization. COS7 cells were transfected with plasmid DNAs containing HA-WT (0.3 μg/10 cm plate) or HA-A^{305} (1.5 μg/10 cm plate) receptors. Transfection with these DNA amounts resulted in similar levels of cell surface expression of HA-WT and HA-A^{305} receptors. At 60 min after 10^{-5} M histamine administration, specific anti-HA antibody binding on COS7 cells expressing the HA-WT receptors decreased by approx. 30%, with a $t_{1/2}$ of 15 min (Fig. 5). In contrast, the total amount of receptor expression was unchanged after a 60 min incubation with 10^{-5} M histamine (not shown). These observations revealed HA-WT recep-

tors to be internalized from the cell surface. A similar decrease in anti-HA antibody binding was observed in HA-A^{305} cells (Fig. 5). In addition, the total amounts of HA-A^{305} receptors expressed were not altered by incubation with 10^{-5} M histamine for 60 min (not shown). Although the total amounts of HA-A^{305} receptors expressed did not correlate with the amounts of cell surface HA-A^{305} receptors as demonstrated by immunocytochemistry (Fig. 2A), cell surface HA-A^{305} receptors appeared to be internalized. Thus, palmitoylation of H2R is not essential for agonist-induced internalization.

Finally, we examined the role of palmitoylation of the receptor in agonist-induced downregulation. To this end, we utilized CHO cell lines stably expressing WT or the A^{305} receptors (termed CHO-WT and CHO-A^{305}, respectively), because the rate of receptor synthesis was not constant in a transient expression system using COS7 cells. These cell lines expressed similar amounts of CHO-WT and CHO-A^{305} receptors as determined by tiotidine binding (Fig. 6A). Each cell line was incubated in the presence or absence of 10^{-5} M histamine for 1, 6, 12 and 24 h. Membrane fractions were prepared and receptor contents, which represent total receptor amounts, were determined by tiotidine binding. Fig. 6A shows that incubation of CHO-WT cells with 10^{-5} M histamine for 24 h resulted in significant reductions in receptor amounts, without altering tiotidine affinity. An approx. 50% decrease in receptor contents was observed, indicating downregulation. Interestingly, incubation of CHO-A^{305} cells under the same conditions resulted in a further reduction in receptor amounts (Fig. 6A). A 70% decrease in receptor contents was observed. Time courses of alterations in all receptor amounts are shown in Fig. 6B. These findings suggest the A^{305} receptor to be more prone to downregulation than the WT receptor. Thus, palmitoylation of H2R might be important for its stability.

4. Discussion

In this study, using a baculovirus/Sf9 expression system and site-directed mutagenesis, we were able to demonstrate canine H2R to be palmitoylated at Cys^{305}. The primary finding in this study is that the subcellular distribution of the A^{305} receptor differs from that of the WT receptor. A larger portion of A^{305} receptors was trapped intracellularly, while WT receptors were located almost exclusively at the plasma membrane. We observed in a previous study that H2Rs with truncation of 70 C-terminal amino acids (T^{289}) were distributed in a manner similar to that of the A^{305} receptor, while a T^{308} receptor with truncation of 51 C-terminal amino acids was distributed at the plasma membranes [20]. The 70, but not the 51, truncated amino acids of these receptors contained Cys^{305}, such that the T^{289} receptor was palmitoylation-defective. These findings indicate that the differences in subcellular distributions of the A^{305} receptor are not due to the replacement of Cys^{305} by Ala, but rather to the loss of palmitoylation secondary to this replacement. Thus, palmitoylation of H2R is important for its plasma membrane trafficking. Cytoplasmic proteins require lipid modifications, such as *N*-myristoylation, palmitoylation and prenylation, for membrane anchorage [25]. However, for membrane proteins with transmembrane structures, lipid modification per se is not essential for plasma membrane targeting, because a large number of membrane proteins without palmitoylation are targeted to the plas-

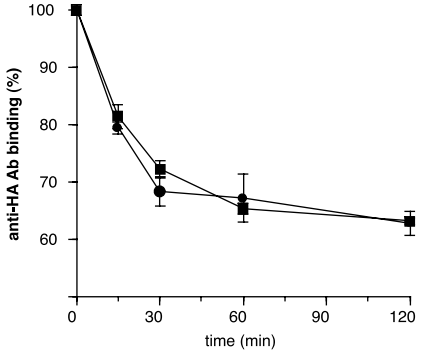

Fig. 5. Effects of histamine treatment on amounts of cell surface receptors or of total receptors. COS7 cells were transfected with plasmid DNAs containing HA-WT (0.3 μg/10 cm plate) or HA-A^{305} (1.5 μg/10 cm plate) receptors. Transfection with these DNA amounts resulted in similar levels of cell surface expression of HA-WT and HA-A^{305} receptors. COS7 cells expressing HA-WT or HA-A^{305} receptors on 24-well plates were incubated with 10^{-5} M histamine or vehicle for the indicated times, and the cells were then fixed in 3% paraformaldehyde/PBS. Cell surface anti-HA antibody binding was determined as described in Section 2. ●, HA-WT; ■, HA-A^{305}. Shown are the means ± S.E. of three individual experiments, each performed in triplicate. When not shown, S.E. values were so small that they fell within the symbols.

ma membrane. Therefore, the effect of the loss of palmitoylation on the subcellular distribution of H2R cannot be explained solely by the lack of palmitoylation.

Because of the different distribution of the A^{305} receptor, it was difficult to compare the function of this receptor with that of the WT H2R. The cell surface A^{305} receptor amount cannot be estimated from the total receptor amount. In addition, there are no hydrophilic radioligands for H2R which can be used to detect cell surface receptors [23]. Thus, we utilized the HA tag and anti-HA antibody to estimate the cell surface receptor amount. We obtained data using this system indicating that lack of palmitoylation did not affect cAMP production via H2R. Rather, the A^{305} receptor was more potent than WT H2R in terms of stimulating adenylyl cyclase. For the α_{2A}-adrenoceptor, loss of palmitoylation did not affect functional coupling to G proteins [2]. Similarly, arginine vasopressin V2 receptors, which were mutated so as to be palmitoylation-defective, were also functionally equivalent to WT in terms of adenylyl cyclase activation [7]. The mutant β_2-adrenoceptor which is devoid of a palmitoylation site showed a reduced ability to mediate isoproterenol stimulation of adenylyl cyclase [1]. However, this is reportedly the result of increased accessibility of the C-terminal portion to receptor kinases rather than lack of palmitoylation [12]. Indeed, chemical depalmitoylation of the β_2-adrenoceptor by hydroxylamine did not affect adenylyl cyclase activation via the receptor [13]. Taken together, these findings suggest that palmitoylation of GPCRs per se is not important for G protein coupling.

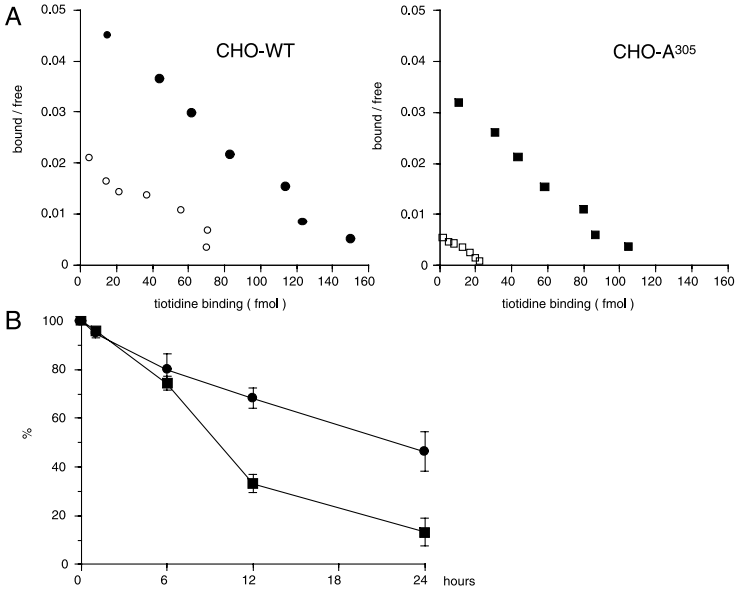

Fig. 6. Effects of prolonged histamine incubation on total receptor amounts. (A) Scatchard plot analysis of tiotidine binding to membranes from treated and untreated cells. After incubation in the presence or absence of 10^{-5} M histamine for 24 h, CHO-WT and CHO-A^{305} cells were washed with PBS three times and membrane fractions were prepared. Membrane fractions (200 μg) from these cells were incubated with 1 nM [^{3}H]tiotidine and varying concentrations of unlabeled tiotidine in 25 mM HEPES, 0.1% BSA, pH 7.4, in a final volume of 200 μl at 37°C for 2 h. The binding reaction was terminated by filtration over GF/C glass fiber filters, followed by 10 ml washes with ice-cold incubation buffer. Radioactivity on the filters was determined by liquid scintillation counting. Specific binding was calculated by subtraction of the non-specific binding determined in the presence of 10^{-4} M ranitidine. Left panel, CHO-WT cells, incubation in the presence (○) or absence (●) of histamine. Right panel, CHO-A^{305} cells, in the presence (□) or absence (■) of histamine. Data shown are representative of three experiments performed in duplicate. (B) Time courses of alterations in B_{max} of tiotidine in membrane fractions from treated cells. CHO-WT and CHO-A^{305} cells were incubated in the presence or absence of 10^{-5} M histamine for 1, 6, 12 and 24 h. Following washes with PBS, membranes were prepared and the B_{max} of tiotidine was determined. The B_{max} of tiotidine in membranes from treated cells was expressed as a percentage of that from untreated cells. Note that the B_{max} of tiotidine in membranes from untreated cells remained constant throughout the incubation period. Shown are the means ± S.E. of three individual experiments, each performed in duplicate. ●, CHO-WT; ■, CHO-A^{305}

In addition, we showed in this study that neither desensitization nor agonist-induced internalization of H2R is affected by the loss of palmitoylation. Since palmitoylation may function in membrane anchorage, creating a fourth intracellular loop, it is conceivable that the conformation of the C-terminus of the A^{305} receptor is different from that of WT. In a previous study, we showed the C-terminus of H2R to be important for agonist-induced internalization, but not for desensitization of the cAMP response [20]. Internalization of the A^{305} receptor also indicates that the presence, rather than proper placement, of the C-terminus, is important for the H2R internalization process. Furthermore, it is noteworthy that the A^{305} mutant, the subcellular distribution of which is completely different from that of WT, was still capable of internalization. This finding indicates that palmitoylation of H2R is involved in subcellular distribution, but not in agonist-induced internalization.

It is widely accepted that cAMP responses occurring via H2R are rapidly desensitized after histamine stimulation [17,20,26–29]. In this study, histamine-dependent cAMP responses via the A^{305} receptor were rapidly desensitized, to an extent comparable to those via the WT receptor, indicating that palmitoylation is not essential for desensitization of the cAMP response. This finding is consistent with our previous observation that truncation of the 51 C-terminal amino acids (which corresponds to the distal portion of the palmitoylation site) of H2R did not affect agonist-induced desensitization of the cAMP response, suggesting that the residues important for desensitization reside in other intracellular loops or in the amino acid residues proximal to Cys^{305} [20]. In the case of the β_2-adrenoceptor, the C-terminus of which is involved in desensitization, lack of palmitoylation led to increased phosphorylation of this region and uncoupling from G proteins [12,13]. This occurs because loss of palmitoylation alters the different conformation of the C-terminus, making it more accessible to receptor kinases.

Finally, we have demonstrated in this study that palmitoylation of H2R is not essential for agonist-induced downregulation. Rather, the A^{305} receptor was found to be more prone to downregulation. In contrast, Eason et al. reported that loss of palmitoylation in the α_{2A}-adrenoceptor abolished agonist-induced downregulation [11]. The roles of palmitoylation in downregulation might differ among receptors. At any rate, it is noteworthy that A^{305} receptors, large numbers of which were distributed intracellularly, were downregulated more markedly than WT receptors. This finding clearly demonstrates that intracellular A^{305} receptors were also affected by 10^{-5} M histamine incubation. There are two possible explanations for this finding. First, it is possible that A^{305} receptors circulate between the plasma membrane and cytoplasm, and when these receptors happen to reach the plasma membrane they bind to histamine. Alternatively, signaling via cell surface A^{305} receptors might have downregulated intracellular A^{305} receptors. In either case, we can conclude that intracellular A^{305} receptors are regulated dynamically.

In conclusion, we have demonstrated in this study that H2R is palmitoylated at Cys^{305} in the cytoplasmic C-terminal region. Furthermore, palmitoylation is important for plasma membrane trafficking but not for H2R functions, such as ligand binding, adenylyl cyclase stimulation, desensitization of cAMP production, agonist-induced internalization and agonist-induced downregulation.

References

[1] B.F. O'Dowd, M. Hnatowich, M.G. Caron, R.J. Lefkowitz, M. Bouvier, J. Biol. Chem. 264 (1989) 7564–7569.
[2] M.E. Kennedy, L.E. Limbird, J. Biol. Chem. 268 (1993) 8003–8011.
[3] G.Y. Ng, B.F. O'Dowd, M. Caron, M. Dennis, M.R. Brann, S.R. George, J. Neurochem. 63 (1994) 1589–1595.
[4] G.Y. Ng, S.R. George, R.L. Zastawny, M. Caron, M. Bouvier, M. Dennis, B.F. O'Dowd, Biochemistry 32 (1993) 11727–11733.
[5] N. Kawate, K.M. Menon, J. Biol. Chem. 269 (1994) 30651–30658.
[6] S. Alaluf, E.R. Mulvihill, R.A. McIlhinney, J. Neurochem. 64 (1995) 1548–1555.
[7] R. Schulein, U. Liebenhoff, H. Muller, M. Birnbaumer, W. Rosenthal, Biochem. J. 313 (1996) 611–616.
[8] A. Horstmeyer, H. Cramer, T. Sauer, W. Muller-Esterl, C. Schroeder, J. Biol. Chem. 271 (1996) 20811–20819.
[9] Z. Gao, Y. Ni, G. Szabo, J. Linden, Biochem. J. 342 (1999) 387–395.
[10] H. Jin, Z. Xie, S.R. George, B.F. O'Dowd, Eur. J. Pharmacol. 386 (1999) 305–312.
[11] M.G. Eason, M.T. Jacinto, C.T. Theiss, S.B. Liggett, Proc. Natl. Acad. Sci. USA 91 (1994) 11178–11182.

[12] S. Moffett, B. Mouillac, H. Bonin, M. Bouvier, EMBO J. 12 (1993) 349–356.
[13] S. Moffett, L. Adam, H. Bonin, T.P. Loisel, M. Bouvier, B. Mouillac, J. Biol. Chem. 271 (1996) 21490–21497.
[14] Y. Fukushima, Y. Ohmachi, T. Asano, M. Nawano, M. Funaki, M. Anai, T. Ogihara, K. Inukai, Y. Onishi, H. Sakoda, T. Saitoh, N. Matsuhashi, Y. Yazaki, K. Sugano, Digestion 60 (1999) 522–527.
[15] I. Gantz, G. Munzert, T. Tashiro, M. Schäffer, L. Wang, J. DelValle, T. Yamada, Biochem. Biophys. Res. Commun. 178 (1991) 1386–1392.
[16] I. Gantz, M. Schäffer, J. DelValle, C. Logsdon, V. Campbell, M. Uhler, T. Yamada, Proc. Natl. Acad. Sci. USA 88 (1991) 429–433.
[17] Y. Fukushima, Y. Oka, H. Katagiri, T. Saitoh, T. Asano, H. Ishihara, N. Matsuhashi, T. Kodama, Y. Yazaki, K. Sugano, Biochem. Biophys. Res. Commun. 190 (1993) 1149–1155.
[18] Y. Fukushima, Y. Oka, T. Saitoh, H. Katagiri, T. Asano, N. Matsuhashi, K. Takata, E. van Breda, Y. Yazaki, K. Sugano, Biochem. J. 310 (1995) 553–558.
[19] H. Otsuka, Y. Fukushima, M. Tamai, H. Takahashi, H. Mori, T. Asano, T. Katsube, K. Ogawa, T. Kajiwara, S. Ohkawa, T. Saitoh, Dig. Dis. Sci. 45 (2000) 796–801.
[20] Y. Fukushima, T. Asano, K. Takata, M. Funaki, T. Ogihara, M. Anai, K. Tsukuda, T. Saitoh, H. Katagiri, M. Aihara, N. Matsuhashi, Y. Oka, Y. Yazaki, K. Sugano, J. Biol. Chem. 272 (1997) 19464–19470.
[21] Y. Fukushima, T. Asano, T. Saitoh, M. Anai, M. Funaki, T. Ogihara, H. Katagiri, N. Matsuhashi, Y. Yazaki, K. Sugano, FEBS Lett. 409 (1997) 286–287.
[22] D.R. Nussenzveig, M. Heinflink, M.C. Gershengorn, J. Biol. Chem. 268 (1993) 2389–2392.
[23] G.A. Gajtkowski, D.B. Norris, T.J. Rising, T.P. Wood, Nature 304 (1983) 65–67.
[24] A. Iida-Klein, J. Guo, L.Y. Xie, H. Juppner, J. Potts Jr., H.M. Kronenberg, F.R. Bringhurst, A.B. Abou-Samra, G.V. Segre, J. Biol. Chem. 270 (1995) 8458–8465.
[25] P.B. Wedegaertner, P.T. Wilson, H.R. Bourne, J. Biol. Chem. 270 (1995) 503–506.
[26] A. Prost, S. Emani, C. Gespach, FEBS Lett. 177 (1984) 227–230.
[27] N. Arima, Y. Kinoshita, A. Nakamura, Y. Yamashita, T. Chiba, Am. J. Physiol. 265 (1993) G987–G992.
[28] Y. Fukushima, T. Asano, H. Katagiri, M. Aihara, T. Saitoh, M. Anai, M. Funaki, T. Ogihara, K. Inukai, N. Matsuhashi, Y. Oka, Y. Yazaki, K. Sugano, Biochem. J. 320 (1996) 27–32.
[29] M.J. Smit, R. Leurs, S.R. Shukrula, A. Bast, H. Timmerman, Eur. J. Pharmacol. 288 (1994) 17–25.